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RESEARCH ARTICLE

Molecular Characterization and Antimicrobial Sensitivity of Pathogens from Sub-Clinical and Clinical Mastitis in Eastern China

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ABSTRACT

Prevalence of sub-clinical and clinical coliform mastitis with antimicrobial sensitivity profile of various mastitis-causing organisms was investigated. Milk samples collected from 299 cows infected with clinical mastitis to evaluate the prevalence of coliform mastitis and 1660 quarters milk samples randomly collected from 415 lactating cows for detection of subclinical mastitis (SCM) by Hangzhou Mastitis Test (HMT). SCM at quarters and cow level was recorded to be 20.2 and 52.3%, respectively. Occurrence of SCM in left rear quarter was high (26.7%). Statistical analysis of risk factors showed, cows with 6-9 years of age (P=0.046; Odds ratio (OR), ±1.414; 95% confidence interval (CI)=1.006-1.988 and 60.7%), cows with 4-7 calves (P=0.028; OR, ±1.502; 95% CI=1.044-2.160 and 62.2%), and cows in late stage of lactation (P=0.039; OR, ±1.947; 95% CI=1.023-3.702 and 68%), were more susceptible to SCM. All the 115 organisms from SCM milk samples and 103 *Escherichia coli* from CM samples were confirmed by PCR techniques. Minimum inhibitory concentration (MIC) results revealed that *E. coli* isolates were resistant to penicillin group (93-99%), fluoroquinolones (40-74%), cephalosporins (54-66%), oxytetracycline (91%), gentamycin (82%), SUL-TRM (88%) and were sensitive to florfenicol. *Staphylococcus aureus* isolates were resistant to ampicillin (91%), oxytetracycline (59%) and methicillin (29%). *Streptococcus agalactiae* isolates were 8 to 15% resistant to used antimicrobials. In conclusion, cows with SCM were reservoir of various bacterial pathogens and high prevalence of *E. coli* in clinical mastitis milk could be major complications for mastitis treatment due to their multidrug resistance profile.

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INTRODUCTION

Mastitis is the inflammation of mammary gland and is a complex and costly disease in dairy herds (Beheshti *et al.,* 2010; Hussain *et al*., 2012a; Atasever, 2012). The main etiological agents causing sub-clinical and clinical mastitis (CM) are *S. aureus*, *Strep. agalactiae* and *E. coli*. However, *E. coli* is reported as most common pathogen causing bovine mastitis subsequent to *S. aureus* (Bansal *et al*., 1990). *E. coli* has importance due to its ability to onset severe infection and rapid acquisition of resistance against commonly used antimicrobials. Severity of coliform mastitis in cattle varies from mild to severe inflammatory and degenerative changes mammary glands (Wenz *et al*., 2006; Hussain *et al*., 2012b). In some countries the prevalence of coliform bovine mastitis are increasing and the control of coliform mastitis is a critical issue due to emergence of *E. coli* as multiple drug resistant (Sumathi, 2008).

Subclinical mastitis (SCM) has a tendency to persist because usually it remains undetected, which results in high milk somatic cell count (SCC) and reduced milk production, treatment cost and development of clinical mastitis and leads to heavy economical losses. About 70 to 80% of the estimated $140 to $300 loss per cow per year from mastitis relates to decreased milk production caused by asymptomatic subclinical mastitis (Leitner *et al*., 2011). The bacterial contamination of milk from the affected cows makes it unhealthy for human consumption and has zoonotic importance (Sharif *et al*., 2009). Keeping view huge economic losses due to SCM and increasing coliform mastitis, this study was carried out to assess the

prevalence of SCM and clinical coliform mastitis with antimicrobial susceptibility test of isolated pathogens, to our knowledge, this is reported first time in eastern China. Our results will be helpful in understanding the risk factors associated with SCM and in proper selection of antimicrobials for the treatment of mastitis infection in this region of China.

MATERIALS AND METHODS

Study AREA and tropical condition: A total of 22 commercial dairy farms in subtropical region of eastern China were selected for this study (Table 1). Average rainfall in this area is 1100mm/year and annual average temperature is 11-19°C. The selected dairy farms are practicing commercial milk production system. The duration of collecting milk samples was about 9 months (March to November 2011). Considering the risk factors for SCM including age, parity and lactation stage of cow, the necessary data were obtained from available farm record. Cows were categorized by age (3-5, 6-9 and 10 years), parity (with 1-3 calves, with 4-7 calves, with 7 calves), and lactation stage (early, mid, and late). All the studied cows examined for clinical mastitis by visible clinical signs and clinical mastitis milk samples were taken to evaluate the prevalence of coliform mastitis in this region.

Table 1: Prevalence of subclinical mastitis at different farms in eastern China

Herd location/ Farms	No. of farms	Total examined	Positive	Prevalence %	95% CI Lower-upper
Nanjing	7	115	67	58.2	0.839-1.934
Taizhou	1	23	11	47.8	0.361-1.938
Xuzhou	1	27	15	55.5	0.521-2.496
Huainan	1	25	14	56	0.515-2.618
Lianyungang	1	20	9	45	0.303-1.839
Weifang	4	75	31	41.3	0.391-1.058
Zaozhuang	1	25	12	48	0.375-1.889
Hangzhou	2	34	20	58.8	0.461-2.650
Nanping	1	18	10	55.5	0.441-2.947
Fengyang	2	38	20	52.6	0.521-1.972
Nanchang	1	15	8	53.3	0.371-2.928
Overall	22	415	217	52.2	

Milk sample collection: Milk samples were collected aseptically from 714 cows (415 lactating cows and 299 clinical mastitis affected cows). SCM was detected using Hangzhou mastitis test (HMT) and results were interpreted according to Hu *et al.* (1990). The quarter milk samples diagnosed with HMT (++ and +++) scores and clinical mastitis milk samples were kept in icebox until transported to laboratory for further investigations. Briefly, before sampling the udder of the cow washed thoroughly and dried with a clean towel. The teats were disinfected with swabs soaked in 70% ethyl alcohol. After discarding first few streams, 7-8 ml milk samples collected in sterile caped tubes and numbered.

Isolation of mastitis pathogens: 30µl of milk sample was added in 3ml nutrient broth (Oxoid UK), and placed in incubator shaker at 37°C for 14-16 hours to obtain the bacterial culture. Phenotypical morphology of creamy white colonies and hemolysis were evident for *Staphylococci* on 5% sheep blood agar base, and dark pink to red colonies on MacConkey's agar (Aoboxing biotech, China) were considered as *E. coli* and other gram-negative organism, bacterial isolates were also identified by gram-staining reaction and morphology. The suspected colonies of *E. coli* and *S. aureus* were further examined on specific media Chrom agar (Biocell Biotech, China) and Baird Parkar agar base (Qingdao Hope bioTech China), respectively. After 24 hours incubation the growing blue colonies on Chrom agar plates were evident for *E. coli* and black colonies with round transparent circle on Baird Parkar agar plates confirmed the growing organism as *S. aureus,* as mentioned by manufacturers. Biochemical tests for identification of *E. coli* were performed according to standard method described previously (Koneman *et al.,* 1997). Identification of *Strep. agalactiae* will be reported later on by another member of our research group.

Confirmation of pathogens by PCR: The isolated pathogens in present study were confirmed by PCR. Genomic DNA was extracted by using DNA purification kit (Geneaid Biotech, Taiwan). DNA templates were amplified with eubacterial primers specific for 16SrDNA gene (1520bp), containing oligonucleotide, FD1 (5'-AGAGTTTGATCCTGGCTCAG -3') and RD1 (5'-AAGGAGGTGATCCAGCC-3'), (Weisburg *et al.,* 1991). Simple PCR used to amplify the 16S rDNA gene, amplification parameters were: initial denaturing step of 5 min at 94°C, following 30 cycles, each consist of 1min at 94°C, annealing 1min at 55°C and extension at 72°C for 1min and a final extension at 72°C for 10min.

PCR products were sequenced from Invitrogen Corp. (Shanghai China). The obtained gene sequences were blasted with National Center for Biotechnology Information (NCBI, http://www. ncbi.nlm.nih.gov). Blast result confirmed 103 isolates as *E. coli* from CM and 115 different isolates from SCM bacteriological culture (data not shown). The relationships of obtained sequences were compared with *E. coli* sequences available at NCBI database. Multiple alignments were calculated by Clustal X program and phylogenetic tree was made by Mega5.05 software, using UPGMA tree method with 500 bootstrap replicates (Fig. 1).

Antimicrobial susceptibility: Antimicrobial susceptibility of isolates was determined by standard broth dilution method on Muller–Hinton (MH) medium (Oxide, UK). For antibiotic sensitivity test of *Strep. agalactiae,* 5% calf serum was added to MH broth. MIC results were interpreted according the Clinical Laboratory Standards Institute (CLSI), standards (CLSI, 2010). Concentration of 1280µg/ml for 12, 10, 8 and 9 antimicrobials agents (Sigma, USA) used in this experiment for *E. coli, S. aureus, Strep. agalactiae* and gram-negative pathogens, respectively. ATCC 25922 was used as quality control strain for MIC determination of *E. coli* and other gram negative isolates, whereas, ATCC 29213 was used as quality control strain for MIC determination of both *S. aureus* and *Strep. agalactiae*.

Statistical analysis: Prevalence of subclinical mastitis and its associated risk factors were determined as the proportion of affected cows out of the total examined. These data were analyzed by descriptive statistics using

the SPSS 11.5 statistical package (SPSS 2002). To assess the role of the various risk factors in occurrence of SCM, we performed chi-square ($\chi 2$) test using the cross tabulation feature of the software. Results considered as significant when $P<0.05$.

RESULTS

In this study, the prevalence of subclinical mastitis at quarters and cow level was 20.2 and 52.3%, respectively. Prevalence of coliform (*E. coli*) mastitis was 34.4%. High prevalence of subclinical mastitis was at Hongzhou and Nanjing farms and lowest prevalence was found at the dairy farms in Weifang (Table 1). Individual quarter level prevalence of subclinical mastitis was higher in left rear-quarter (LR) (Table 2).

Investigated risk factors and their possible association with subclinical mastitis on dairy farms of this region are shown in Table 3. Chi squared analysis indicated that all the evaluated risk factors showed significant association with occurrence of SCM. The cows in age group 6-9 years were significantly susceptible to SCM (P=0.046). Likewise, high frequency of SCM observed in cows with 4-7 calves (P=0.028). Cows at late stage of lactation were significantly susceptible to SCM (P=0.039).

Table 2: Prevalence of subclinical mastitis at quarter level

Quarter	No. Positive	Total examined	Prevalence %	95% CI Lower-upper
RF	54	415	13	0.432-0.804
LF	91	415	21.9	0.852-1.438
RR	80	415	19.2	0.717-1.235
LR	111	415	26.7	1.123-1.844
Total	336	1660	20.2	

Table 3: Subclinical mastitis associated risk factors (age, parity and stage of lactation).

Parameters	Positive		Negative	Chi-sq	Odds	95% CI
	N	%		*P* value	ratio (OR)	Lower-upper
Age group						
3-5 years	77	42.0	106	0.021	0.663	0.467-0.942
6-9 years	124	60.7	80	0.046	1.414	1.006-1.988
□10 years	16	57.1	12	0.619	1.217	0.562-2.635
Parity						
1-3	98	43.5	127	0.035	0.704	0.508-0.976
4-7	107	62.2	65	0.028	1.502	1.044-2.160
□7	12	66.6	6	0.232	1.825	0.672-4.954
Lactation stage						
Early	111	61.6	69	0.035	1.468	1.027-2.097
Mid	74	39.3	114	0.003	0.592	0.417-0.841
Late	32	68.0	15	0.039	1.947	1.023-3.702

Totally, 115 bacterial pathogens identified from SCM positive quarter milk samples. The *S. aureus* was the most prevalent pathogen (29.5%) amongst all isolated organisms, followed by *Strep. agalactiae* (22.6%), *Klebsiella* (9.5%), *Anterobacter* (7.8%), *Aeromonas* (6.9%), *Pseudomonas* (6%), *Streptococcus uberis* (5.2%), *Citrobacter* (4.3%), *Pantoea* (4.3%), *Staphylococcus hyicus* (1.7%) and *Streptococcus dysgalactiae* (1.7%).

Of 299 clinical mastitis infected cows, 103(34.4%) were infected with coliform mastitis. High prevalence of coliform mastitis was on Taizhou, Huainan and Nanping farms (40%), followed by Hangzhou (38%), Nanjing (37.5%), Fengyang (33.3%), Zaozhuang (33.3%), Xuzhou (30.7%), Lianyungang and Nanchang (30%) and (20.5%) at Weifang dairy farms.

MIC results showed *E. coli* isolates were highly resistant to almost all tested antimicrobials commonly used in veterinary practice. Whereas, all the *E. coli* isolates were sensitive to florfenicol (Fig. 2).

S. aureus isolates were sensitive to cefquinome but were highly resistant to penicillin, ampicillin, oxytetracycline and methicillin. A very less percentage of *Strep. agalactiae* isolates showed resistance to the tested antimicrobials. Other gram-negative isolates showed different patterns of resistance against tested antimicrobials, except *Klebsiella* all gram-negative isolates were sensitive to florfenicol and enrofloxacin (Table 4).

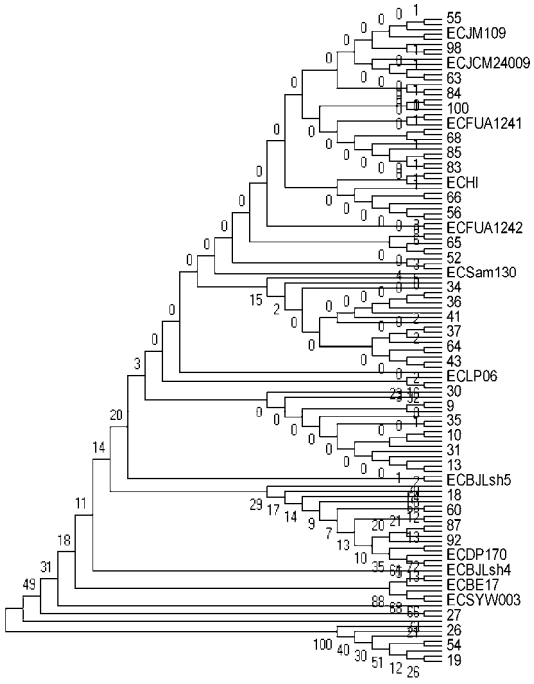

Fig. 1: Phylogenetic tree showing relationship among obtained sequences of our *E. coli* isolates with the available sequences of *E. coli* in NCBI database.

DISCUSSION

Most of clinical mastitis cases are preceded by sub-clinical mastitis and leading to chronic form of disease. SCM mastitis related annual economic losses in dairy industry of USA and Europe are more then 2 billion dollars which are attributable to reduced milk quality, early culling, treatment costs, veterinary expenses, and increased labor costs (Leitner *et al*., 2011; Hussain *et al*., 2012c). In China, 40-80% milking cows are affected with SCM (Zhang *et al.*, 2005). In present study, SCM results showed 52.2% animals with 20.2% quarter infection rate are in agreement with those of Li *et al*. (2009), who reported 54.3 and 28.0% at cow and quarter level, respectively in Zhejiang province of China. High prevalence of SCM in left rear quarter is in disagreement with previous report (Shittu *et al*., 2012), which showed the prevalence of SCM in left fore quarter. In our study, a significant influence of age and parity on subclinical mastitis was observed, high prevalence of SCM with increased age and parity is in accordance with previous studies (Haftu *et al*., 2012). It is established that increasing age and number of parity keeps the teat canal

Table 4: Minimum inhibitory concentration results of isolates from subclinical mastitis showing resistance (%)

Pathogen	No. of Isolates	PEN-G	AMP	OX	CEFQ	CIP	ENR	TRM	MTH	CMP	OTC
S. aureus	34	47	91	9	SEN	26	3	56	29	32	59
		P/NB	AMOX	AMP	CEFT	VAN	CMP	OFLOX	LEVO	-	-
S. agalactiae	26	15	8	12	8	11	12	12	8	-	-
		AMP	CEF	CIP	DANO	ENR	FFC	GEN	TC	TRM	-
Enterobacter	9	44	33	22	22	SEN	SEN	55	55	33	-
Citrobacter	5	60	20	20	20	SEN	SEN	40	60	40	-
Klebsiella	11	45	18	36	36	9	SEN	54	55	27	-
Pantoea	5	60	20	40	20	SEN	SEN	SEN	20	20	-
Pseudomonas	7	56	14	28	28	SEN	SEN	28	42	28	-
Aeromonas	8	50	25	25	25	SEN	SEN	37	62	25	-

PEN-G: penicillin G, AMP: ampicillin, OX: oxacillin, CEFQ: cefquinome, CIP: ciprofloxacin, ENR: enrofloxacin, TRM: trimethoprim, MTH: methicillin, CMP: chloramphenicol, OTC: oxytetracycline, P/NB: penicillin novobiocin, AMOX: amoxicillin, CEFT: ceftriaxone, VCM: vancomycin, CMP: chloramphenicol, OfLOX: ofloxacin, LEVO: Levofloxacin, CEF: ceftiofur, DANO: danofloxacin, FFC: florfenicol, GEN: gentamicin and TC: tetracycline.

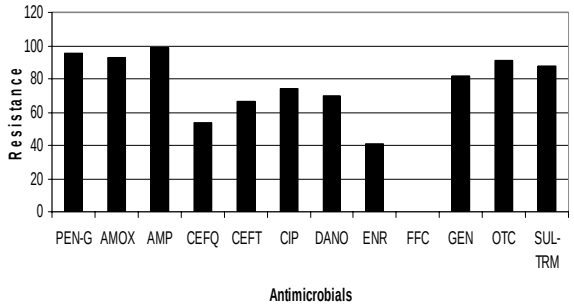

Fig. 2: Resistance percentage of various antimicrobial drugs to *E. coli* isolated from clinical mastitis cases (PEN-G: Penicillin G, AMOX: amoxicillin, AMP: ampicillin, CEFQ: cefquinome, CEF: ceftiofur, CIP: ciprofloxacin, DANO: danofloxacin, ENR: enrofloxacin, FFC: florfenicol, GEN: gentamicin, OTC: oxytetracycline and SUL-TRM: sulfadiazine and trimethoprim).

permanently dilated due to repeated milking and lactations, thus facilittating the enrty of pathogens into the teat canal and causing subclinial intramammary infection (Shittu *et al*., 2012). Some authors have reported that the cows at early stage of lactation are more susceptible to SCM (Kerro and Tareke, 2003). Conversely, other studies reported mid and late stages of lactation as high risk for SCM (Getahun *et al*., 2008; Haftu *et al*., 2012). We also found that the late and early stages of lactation were significantly associated with occurrence of SCM ($P<0.05$). The difference in the effect of stage of lactation reported in many studies is attributed to the increased opportunity of infection with time and the prolonged duration of infection, difference in age, parity and breed of the investigated animals.

Among all the identified pathogens from SCM, *S. aureus* was the most prevalent (29.5%) and is similar to previously reported 32.1% in China (Feng-Li, 2011) but higher than 10-15% prevalence in SCM reported by Liu *et al.* (2007). *S. aureus* considered as a cause of subclinical chronic infections and could be a source of spreading the infections to other healthy cows (Workineh *et al*., 2002).

Strep. *agalactiae* was recovered from 22.6% SCM affected quarters, its prevalence is higher than 17.9% reported by Feng-Li (2011). Interestingly, no *E. coli* isolate was present in SCM samples, which is contradictory with the 16% prevalence shown by Abdel-Rady (2009). Our findings revealed that *E. coli* is only associated with clinical mastitis infection. Various gram-negative organisms including *Klebsiella* were found in SCM milk samples; this is in accordance with previous reports suggesting that this environmental pathogen can cause IMI (Nam *et al*., 2009; Botrel *et al*., 2010). Prevalence of clinical coliform mastitis was 34.4% in this region, which is greater than recently reported 25.7% by Feng-Li (2011) and 24% reported by Bradley and Green (2001).

Antimicrobial susceptibility results showed multidrug resistance in *E. coli* isolates against all tested antimicrobials except florfenicol, sensitivity to florfenicol might be due to its rare use in cattle. Observed multidrug resistance in *E. coli* isolates is higher than previously reported 20% by Suojala *et al*. (2011). Similarly, *S. aureus* isolates were also multidrug resistant, observed resistance against ampicillin and penicillin is in agreement with Mekonnen *et al*. (2005) and supports the findings of Green and Bradley (2004). All the *S. aureus* isolates were sensitive to cefquinome, whereas 29% isolate were resistant to methicillin which is much lower than 60% reported by Kirkan *et al*. (2005). Methicillin resistant *S. aureus* (MRSA) is a serious threat for human health and gaining high attention due to its zoonotic importance. *Strep. agalactiae* isolates were sensitive to almost all antimicrobial agents except betalactams, our findings are in contrast with Guerin-Faublee *et al*. (2002), who reported all *Strep. dysgalactiae* and *Strep. agalactiae* strains were susceptible to betalactams. Antibiotic resistance in the gram-negative opportunistic pathogens is emerging problem and a greater concern to veterinarians in curbing infection in animals, because gram-negative bacteria can easily transfer the resistance determinants among each other.

Conclusion: The prevalence SCM was moderate and found associated with increased age, number of parity and late stage of lactation. High prevalence of coliform mastitis was observed in this region. Multidrug resistance in *E. coli, S. aureus* and other gram-negative pathogens might be the result of indiscriminate and improper use of antimicrobials. This study reports high prevalence of *E. coli* in clinical mastitic cattle and its absence in healthy cows milk raises a question that *E. coli* may have become equaly responsible for cauisng bovine mastitis like *S. aureus.*

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